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SIGNAL TRASDUCTION OF THE CONSTITUTIVELY ACTIVATED FIBROBLAST GROWTH FACTOR RECEPTOR 3 (FGFR3)

S.S.D. BIO/10 Biochimica

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Riassunto

Fibroblast Growth Factor Receptor 3 (FGFR3) appartiene alla famiglia dei recettori tirosin-chinasici e ha un ruolo importante come regolatore negativo del processo di ossificazione endocondrale.
Mutazioni altamente attivanti di FGFR3 provocano gravi forme di displasie scheletriche.
Durante i miei studi ho considerato se l'organizzazione del citoscheletro potesse essere influenzata della mutazione K650M, associata ad una grave acondroplasia con ritardo nello sviluppo e acantosis nigricans (SADDAN).
FGFR3-SADDAN è un mutante altamente attivato che si trova accumulato in forma immatura glicosilata di 120kDa nel reticolo endoplasmico (ER). Da questo compartimento intracellulare è in grado di segnalare attraverso una via FRS2α e PLCγ indipendente e attivare le ERKs.
Ci siamo chiesti se questo *signalling* anomalo dal reticolo endoplasmico potesse avere un ruolo importante nel determinare il fenotipo patologico del recettore.
I dati che abbiamo ottenuto indicano che FGFR3-SADDAN recluta PLCγ, Pyk2 dal ER.
Ciò si traduce in un aumento della fosforilazione di Pyk2 e paxillina, eventi associati alla disorganizzazione dell'actina citoscheletrica.
Inoltre, durante il mio lavoro, abbiamo scoperto che abrogando l'interazione FGFR3/PLCγ, mediante la sostituzione Y754F in FGFR3-SADDAN, il recettore prosegue il suo *pathway* secretorio e raggiungere la membrana plasmatici, sulla quale è presente nella forma matura di 130kDa, pur rimanendo costitutivamente attivato.
Crediamo che PLCγ giochi un ruolo chiave in quanto, legando FGFR3-SADDAN, impedisce che il recettore completi la sua biosintesi.
Noi ipotizziamo che questo accada perchè si forma una "piattaforma" di segnalazione che coinvolge il recettore mutato e permette un *signalling* anomalo, forzando la permanenza di FGFR3-SADDAN nel reticolo endoplasmico.
Questa via di segnalazione anomala dal ER determina inoltre la disorganizzazione delle strutture citoscheletriche suggerendo che questi eventi inducano displasie scheletriche mediate da FGFR3.

Abstract

Fibroblast growth factor receptor 3 (FGFR3) belong to the tyrosine kinase receptor (RTK) family and plays a pivotal role in skeletal development being a negative regulator of bone growth as target disruption of the mouse FGFR3 gene causes a skeletal overgrowth.

Many other mutations located in different domain of FGFR3 have been associated with skeletal diseases with graded severity, in particular gain-of-function mutation affecting the codon 650 within the critical kinase domain of FGFR3.

The aim of our study was to investigate, in vitro, on the role by a mutant FGFR3 associated to the severe achondroplasia with developmental delay and achanthosis nigricans (SADDAN) on cytoskeletal organization.

The SADDAN mutant revealed the unpaired trafficking of the immature mannose-rich 120kDa SADDAN receptor that remain localized in the ER, and transducers signal in its immature from leading to ERKs activation trough FRS2α and PLCγ-independent pathways.

We have questioned whether the intracellular position of FGFR3 signalling has a critical role on the receptor-induced phenotype.

Our findings indicate that PLCγ, Pyk2, paxillin interact with the immature FGFR3-SADDAN glycomers from the ER.

These events are associated to an increased phosphorylation of paxillin/Pyk2 and the perturbed actin cytoskeltal organization.

Preventing the PLCγ/FGFR3 interaction by the Y754F amino acid substitution in FGFR3 results in the failure of both Pyk2 recruitment and paxillin enhanced phosphorylation and restores the receptor full maturation on cell surface.

We propose that PLCγ through its early engagement with the immature FGFR3-SADDAN confers a functional signalling activity to the receptor thus forcing its permanence in the ER.

Altogether the data presented herein indicate that the interaction between PLCγ and the activated receptor in the ER are key events to determine the FGFR3-SADDAN-perturbed cytoskeletal organization and suggest that actin cytoskeleton is a target for the FGFR3-induced skeletal dysplasias.

Introduction

1.1 Fibroblast Growth Factor Receptor 3 (FGFR3).

Fibroblast growth factor receptors (FGFRs) belong to the tyrosine kinase receptor (RTK) family (1). Fibroblast Growth Factor Receptor 3 (FGFR3) is one of four distinct members of glycosilated transmembrane proteins that serve as high affinity receptors (FGFRs) for more 23 different fibroblast growth factors (FGFs) (1,2,3 and 4).

The four FGFRs consist of independent gene that share a highly conservated structure: an extracelluar domain composed of three immunoglobulin-like domains, a single transmembrane helix idrophobic domain, and an intracellular domain with tyrosine kinase activity (Fig.1).

The three immunoglobulin(Ig)-like domains, D1, D2, and D3, present a stretch of acid amino acid (the acid box) between D1 and D2. This acid box can participate in the regulation of FGF binding to FGFR, each receptor can be active by several FGFs. D2 is the binding site of heparin or heparin sulphate and present a highly conservated structure and positively charge (5).

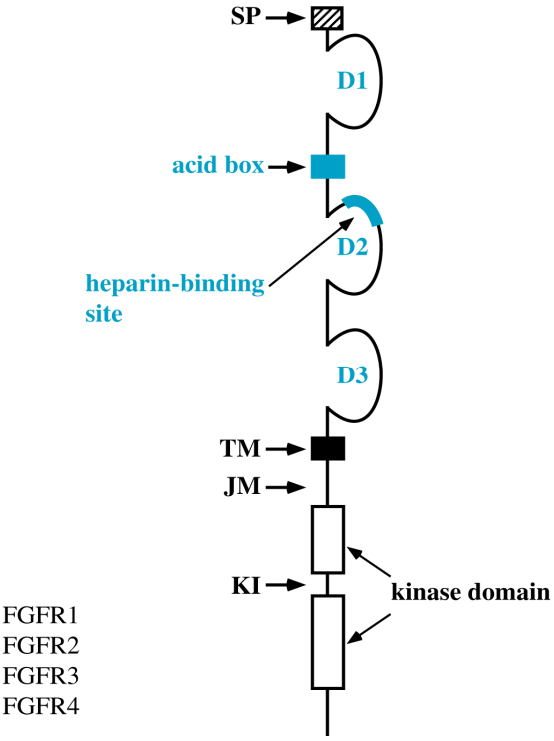

Fig.1
Schematic representation of the FGFRs.
D1, D2 and D3 are three immunoglobulin-like domains.
TM is an idrofobic transmembran region.
KI indicate the two tyrosine kinase domains.

The FGFR3 early biosynthesis is characterized by native un-glycosilated 98kDa protein and the immature mannose-rich 120kDa protein endoplasmic reticulum (ER) resident. The mature 130 kDa fully glycosylated protein is exposed on the cell surface as monomer (5).

Upon binding with FGF ligands and heparin, the receptor dimerizes and undergoes inter-chain autophosphorylation at number of tyrosine residues in the intracellular domain, thus transmitting

signals into the cells (6).

The signalling pathway activated by FGF-stimulated FGFR3 passes through the activation of docking protein FRS2α and subsequently the Grb-Sos recruitment that in turn activates the Ras pathway through Raf and MAPK phosphorylation, ending out ERK1/2 protein activation.

Phosphorilated ERKs translocate to the nucleus where they activate factor playing a major role in gene expression (7).

A second signalling pathway activated by FGF-FGFR interaction involves the recruitment of Phospholipase-C-gamma (PLCγ), which trough the activation of PKC proteins leads to the ERKs phosphorylation (8).

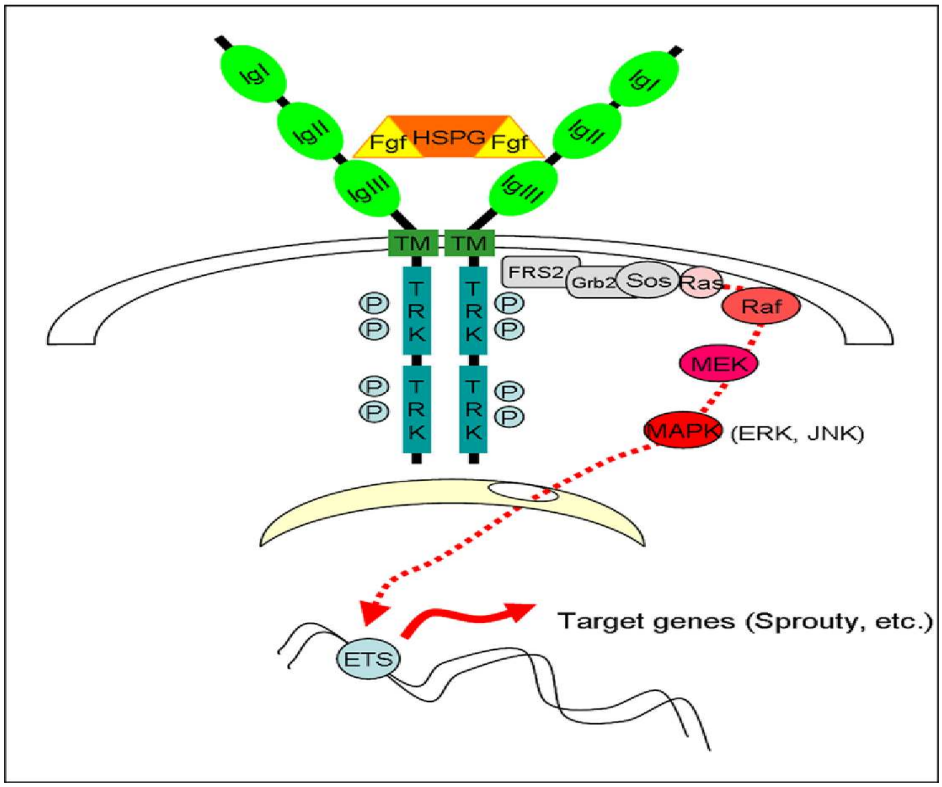

Fig.2 Representation of the signalling pathway active by FGF-stimulated FGFR3.

FGFRs serve as specific receptors for the FGF family of growth factor that mediate important cellular function during development such a differentiation, motility and proliferation.

FGFR3 plays a pivotal role in skeletal development being a negative regulator of bone growth as target disruption of the mouse FGFR3 gene causes a skeletal overgrowth (9, 10).

Many human skeletal disorders have been associated to mutations in the various FGFRs (11).

Mutations in FGFR3 are responsible for disorders predominantly of the long bones, including Achondroplasia (ACH, mutation G308R), the most common from of human genetic dwarfism (12).

Many other mutations located in different domain of FGFR3 have been associated with skeletal diseases with graded severity, in particular gain-of-function mutation affecting the codon 650 within

the critical kinase domain of FGFR3.

FGFR3 activation cause severe skeletal dysplasia's in human like thanatophoric dysplasia type II (TDII, K650E) and the severe achondroplasia with delay in development and acanthosis nigricans (SADDAN,K650M) (13).

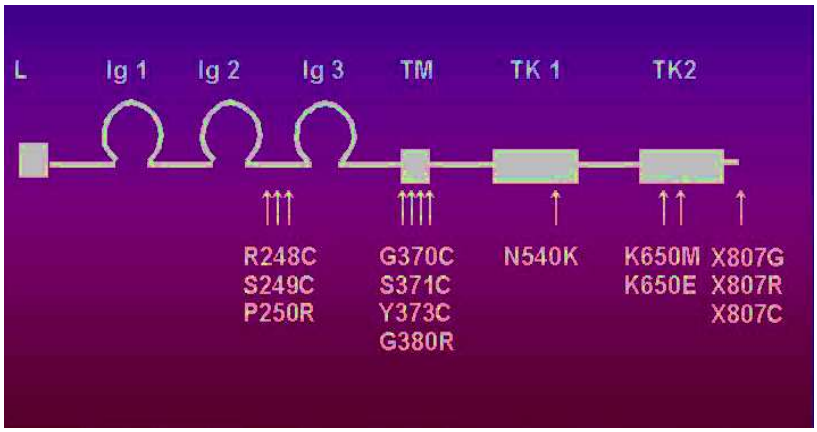

Fig.3
Representation of the FGFR3 with the tyrosine kinase domain (TK1 and TK2) and the Lys-650 codon. Relative substitutions are indicated with the severity of the disease. K650M substitution causes SADDAN disease.

All those disease are associated to dysfunctions at the endochondral ossification, the process responsible for elongation (14). In this process FGFR3 play a key role as negative regulator of chondrocyte proliferation.

The K650M substitution, causing SADDAN lies in the activation loop domain and leads to the constitutive activation of the receptor in the absence of ligand.

Studies aimed to determinate the biochemical features acquired by the SADDAN mutant revealed the unpaired trafficking of the immature mannose-rich 120kDa SADDAN receptor that remain localized in the ER (15).

Furthermore, it has been shown that the FGFR3-SADDAN transduces signal in its immature from leading to STAT1 and ERKs activation (15, 16).

ERKs activation by the ER-trapped SADDAN receptor bypasses the recruitment of FRS2α indicating that a pathway alternative to the canonical cascade is implicated.

FGFR3-SADDAN mutants induce Erk1/2 activation trough FRS2α and PLCγ-independent pathways (17).

1.2 The endochondral ossification.

Endochondral ossification is one of the two essential processes during fetal development of the mammalian skeletal system by witch bone tissue is created.

Unlike intramembranous ossification, which is the other process by which bone tissue is created, cartilage is present during endochondral ossification. It is also an essential process during the rudimentary formation of long bones, the growth of the length of long bones, and the natural healing of bone fractures.

Chondrocytes are the cellular component of cartilage. The development of cartilage template is essential for proper formation of endochondral bones (18).

The process of chondrocytogenesis begins with the stimulation of mesenchymal cells to condensate. Cells within these condensations then differentiate into chondrocytes and start to proliferate: these processes determine the size and location of future skeletal elements (19, 20, and 21).

Chondrocytes in the most central region of the template differentiate to the terminal stage of the hypertrophic chondrocyte (22). Chondrocytes located between the resting/reserve zone and the hypertrophic zone, proliferate in a unidirectional manner, resulting in characteristic columns (23). Hypertrophic chondrocytes mineralize the matrix surrounding them, undergo apoptosis, and hypertrophic cartilage is invaded by blood vessels (24). The mineralized cartilage is degraded by osteoclasts and replaced with bone tissue through the activity of osteoblasts (25, 26). The regions on either side of the bone tissue are termed the growth plates and responsible for longitudinal growth (Fig.4).

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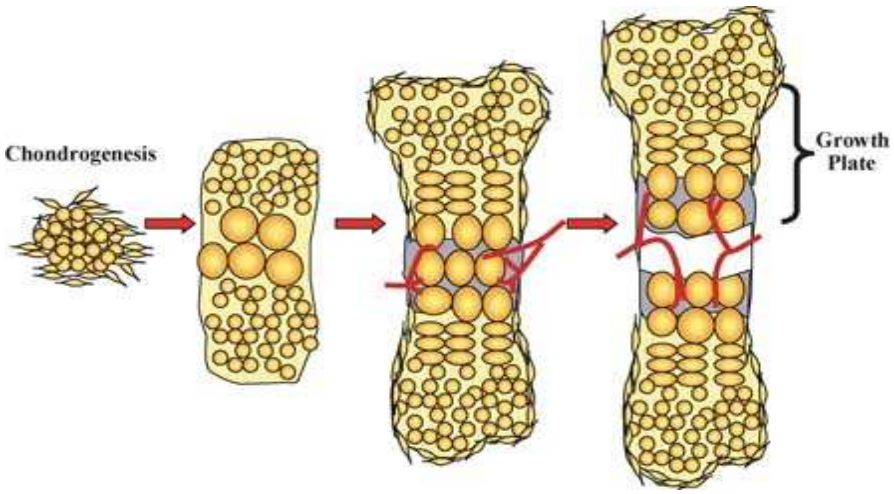

Fig.4 Illustration of endochondral ossification and Growth Plate Development.

1.3 FGFR3 and skeletal dysplasias.

The expression of FGFRs is tightly regulated during embryonal development and tissue regeneration (27, 28, and 29).

FGFR3 is particularly highly expressed during embryonic development in the pre-cartilaginous mesenchyme and later on in the maturation zone of epiphyseal growth-plates, where it is involved in long bone development (30).

FGFR3-null mice exhibit bone overgrowth accompanied by expansion of proliferating and hypertrophic chondrocytes within the growth-plate (9, 10).

The discovery that specific activating mutations in FGFR3 underlie a variety of human skeletal disorder, such as Achondroplasia, the most common form of human genetic dwarfism, has linked FGFR3 signalling and skeletal development (3, 31).

Transgenic mice harbouring FGFR-activating mutations or overexpressing FGF2 or FGF9 (32) display a dwarf phenotype similar to the human disorder where attenuated proliferation and differentiation of chondrocytes result in retarded bone growth (33, 34).

FGFR3 acts as a potent regulator of chondrocyte differentiation and as a negative regulator of bone growth.

1.4 Role of the cytoskeleton in chondrocyte differentiation.

Recent data suggest that actin dynamics not only control chondrogenesis, but also growth plate physiology (35).

Several human chondrodysplasias have been linked to mutation affecting the actin cytoskeleton.

Because of these established role of the actin cytoskeleton, investigations into the upstream regulators of cytoskeletal dynamics in chondrocyte physiology.

Rho GTPases are molecular switches in the cell that activated downstream effectors when bound to GTP and are inactivated by hydrolysis to GDP. The activity is highly regulated by interaction with other proteins and post-translational modifications (36).

Rho family GTPases play pivotal role in reorganization of actin cytoskeleton arrangement such as the formation of stress fiber or membrane ruffles.

Rac1 is a Rho GTPase that promotes chondrogenesis, the differentiation of chondrocyte to hypertrophy and in addition Rac1 signalling inhibit proliferation of chondrocytes when overexpressed in cell culture (37, 38).

Furthmore, cartilage-specific ablation of the Rac1 gene results in shortened long bones and disorganized, hypo-cellular growth plates.

Adhesion receptors such as integrins bind wide range of extracellular components such as ECM molecules and other cell surfaces proteins. Integrin attachment stimulates the formation of focal adhesion complex, an intracellular protein complex that transduces signals from the ECM to intracellular effectors such as cytoskeleton (39, 40).

It should be noted that integrin signalling has been studied extensively in articular chondrocytes and has been implicated in cartilage homeostasis and arthritis (41).

1.5. Integrin-mediated signalling.

Integrins are transmembrane heterodimeric glycoproteins consisting of α and β subunits that mediate cell-cell and cell-extracellular matrix (ECM) interactions (42, 43).

After ECM (fibronectin) stimulation, integrin clustering promotes the recruitment of focal complexes. The direct interaction of PLCγ1 with integrin β1, Src-mediated, activation of PLCγ1 induces the hydrolization of PIP2 to generate IP3 and DAG, which are implicated in the mobilization of intracellular Ca2+ and protein kinase C activation.

They specifically activate Pyk2/paxillin and activated Pyk2 facilitates the activation of Race GTPase, which can regulate cellular adhesion and motility (Fig.5).

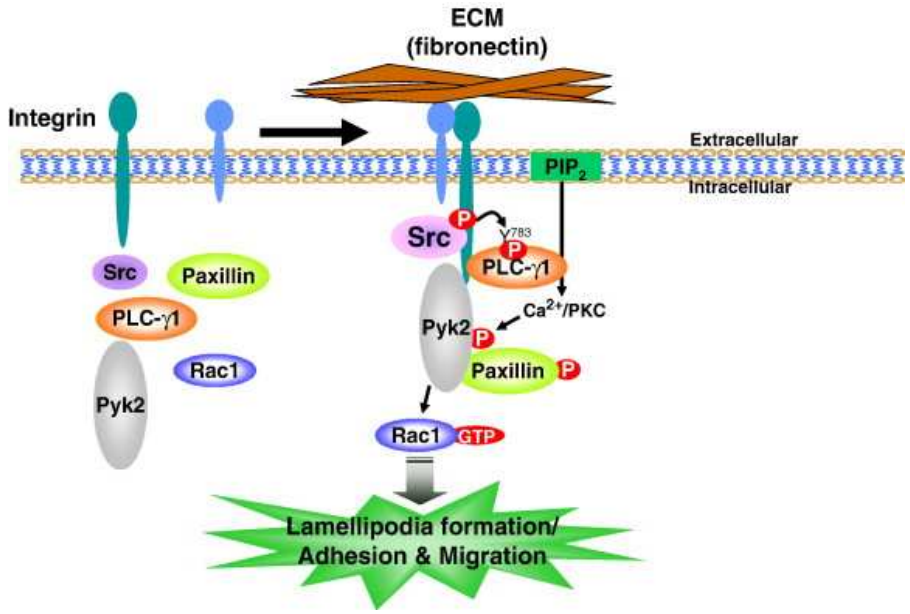

Fig.5 Schematic representation of integrin-mediated signalling.

The initial delay in integrin-stimulated cell spreading by the loss of PLC-γ1 is associated with actin cytoskeletal rearrangement and retarded focal adhesion formation, which are associated with reduced Pyk2/paxillin phosphorylation. Furthermore, there is a long term defect in migration ability after spreading has been completed (44).

Phospholipase C gamma (PLC γ) is involved in cellular proliferation and differentiation, and its enzymatic activity is up-regulated by a variety of growth factors and hormones (45).

Many growth factors such as platelet-derived-growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and nerve growth factor (NGF) elicit tyrosine phosphorylation of PLC-γ1 with stimulation of PIP_2 turnover in a wide variety of cells (46, 47 and 48).

PLC-γ1 modulates cellular adhesion, spreading and migration through the activation of Pyk2/paxillin.

Rational and aim of the study

FGFR3 play a key role as negative regulator of chondrocyte proliferation.

Mutations located in different domains of FGFR3 have been associated with skeletal diseases with graded severity, in particular gain-of-function mutation affecting the codon 650 within the critical kinase domain of FGFR3.

The K650M substitution, causing SADDAN lies in the activation loop domain and leads to the constitutive activation of the receptor in the absence of ligand.

Studies aimed to determinate the biochemical features acquired by the SADDAN mutant revealed the unpaired trafficking of the immature mannose-rich 120kDa SADDAN receptor that remain localized in the ER.

Based on observation that the FGFR3-SADDAN transduces signal in its immature form leading to STAT1 and ERKs activation in the early phase of its biosynthesis, we questioned whether such signalling is the cause by cytoskeletal disorganization.

We hypothesize that abnormal signalling of FGFR3-SADDAN from the ER to the direct recruitment of Pyk2 and paxillin, can disrupt the organization of actin cytoskeleton.

The aim of this study is to determine whether the abnormal signaling of the SADDAN mutant from the ER could be the cause of alterations in cytoskeletal structures and the incomplete biosynthesis of the mutated receptor.

In these studies we use a traditional cell culture DNA transfection approach and biochemical techniques based on co-immunoprecipitation assay using FGFR3 molecules having different mutation.

Then we performed immunohistochemical experiments with confocal microscopy to confirm our data.

Materials and Methods

FGFR3 construct.

Plasmids carrying the murine FGFR3-wt-HA, SADDAN-HA cDNA have been describing (16).The Y754F substitution and the was generated by polymerase chain reaction (PCR) using the following primers:

5′-GAGGAACAGCTCACCTGCA-3′ (mFR3F1),
5′-CAACCGACGAGTTCTTGGACCTCT-3′ (F754mFR3F),
5′-AGAGGTCCAAGAACTCGTCGGTTG-3′ (F754mFR3R),
5′-GCTATTTAGGTGACACTATAG-3′ (SP6a).
The N502A substitution was generated using the following primers:
5′-ACCGTGCACAAGGTCTCTC-3′ (mFR3F4),
5′-ACCGTGGCCGTGGCGATGCTGAAAGA-3′ (A502mFR3F),
5′-TCTTTCAGCATCGCCACGGCCACGGT-3′ (A502mFR3R),
5′-CATTTGTGGTCTTCTTGTAGTA-3′ (mFR3644R).
The general strategy has been described (17).

Cell culture and transfection.

HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum (Euroclone), 2mM L-glutamine and penicillin/streptomycin.
Transient transfections in HEK293 were preformatted with Lipofectamine 2000 (Invitrogen) according to manufacturer's instruction and cells were collected after 48h.

Immunoprecipitation and western blot.

Trasfected cells were lysed in LB4 buffer (50mM Tris-HCl pH=8, 150mM NaCl, 1% Nonidet P-40) in the presence of mixture of protease and phosphatase inhibitors, clarified by centrifugation and subjected to immunoblot; FGFR3 proteins were resolved on 7% tricine gels, paxillin and Rac1 proteins were separated by 8% and 12% tricine gels.
The immunoprecipitation were preformatted from 1mg of total proteins, immunocomplexes were collected with protein A-Sepharose (Amersham Biosciences), washed three times with lysis buffer

and one time with TBS1X (TRIS 100mM, 1,5M NaCl).
Proteins were transferred onto polyvinylidene difluoride membranes (ImmobilonP, Millipore), blocked with 5% milk in Tris-buffered saline, pH 7.5, and incubated with desired antibodies.

Antibodies and inhibitors.

The following antibodies were used: anti-HA (clone 12CA5) (Roche), anti-Phospho tyrosine antibody 4G10 (Upstate) and anti-Phosho-tyrosine agarose conjugated, anti-PLCγ (Cell Signaling), anti-Vinculin (V240, Millipore), anti-Pyk2 (BioLegend), anti-Paxillin (Transduces Laboratories), anti-Phospho paxillin (Cell Signaling).Where indicated cells were treated whit 3mM U73122 or U73433 for 24h (Calbiochem).

Cell surface biotinylation.

Cells were incubated with 400 μg/ml of Biotin X-NHS (Pierce) in phosphate-buffered saline (PBS) for 10 min at 4°C, washed, collected and lysed. The biotinylated proteins were recovered by incubating cell lysates with agarose-conjugated streptavidin (Sigma) while the un-biotinylated FGFR3 isoforms were isolated by subsequent re-immunoprecipitation with anti-HA antibodies. The recovered proteins were analyzed by Western blot with anti-HA antibodies.

Deglycosilation

Immunoprecipitated complexes were resuspended in 50 Mm sodium citrate, pH 5.5, containing 1% SDS and 1% β-mercaptoethanol and boiled for 10 min. Endoglycosidase H (Endo H) (New England Biolabs) was added to the recovered samples, and the reaction was carried out at 37 C° over night. Concerning the PNGaseF (New England Biolabs) reactions, samples were diluted 1:1 with 50 Mm sodium citrate, and 1% of Nonidet P-40 was added prior to adding the enzyme. Both enzymatic reactions were carried out at 37 C° over night.

Immunocytochemistry.

Cells were fixed with 4% (w/v) paraformaldehyde (PFA) for 30 min. PFA was quenched with 50mM NH4Cl. Cells were then permeabilized with PBS-0,1% Triton X-100, blocked with 1%BSA fro 30 min and stained with primary Abs for 1 h. Cells were stained with secondary Abs and

rhodamine-phalloidin for 30 min, followed by DAPI for 10 min. Images were collected using a confocal microscope (Leica SP5) with 63x objective. Images were processed for brightness and contrast with Adobe Photoshop.

Results

2.1 Activated FGFR3-SADDAN mutant triggers the PLCγ/Pyk2 interaction.

As previously described (15), the FGFR3-SADDAN mutant is expressed predominantly in its immature 120kDa species, which is highly phosphorylated and resides in the ER.
FGFR3-SADDAN exhibits signalling activity in the early phase of biosynthesis by directly recruitment both PLCγ and Pyk2.
PLCγ is required for actin cytoskeletal organization and cell motility through the regulation of Pyk2 and paxillin activation (44).
In these aim we have studied whether the abnormal signalling from the ER of the FGFR3-SADDAN mutant could as well affect cytoskeletal organization through PLCγ and Pyk2 regulation.
For this purpose, we have performed immunoprecipitation experiments to determinate whether PLCγ directly interacts whit Pyk2 in cells trasfected with the SADDAN receptor variant.
In addition the wt-754 and SADDAN-754 were also analyzed; the Y753F amino-acid substitution inhibits the binding of the receptor with PLCγ.
As shown in fig.6A, the SADDAN-754 variants as well as both wt and wt-754 do not present Pyk2 recruitment. The immature SADDAN glycomers recruit and phosphorylate PLCγ, suggesting that both PLCγ interaction and kinase activity are conditions required for Pyk2 recruitment from ER.
Accordingly, by re-probing the filter with anti-HA or 4G10 antibody reveal the 120kDa band corresponding to the ER-resident SADDAN mutant is detected and a diffuse band indicative of a series of phosphorylated proteins in correspondence to the SADDAN-transfected cells.
Since activated PLCγ has been shown to enhance the Pyk2 tyrosine phosphorylation, we have determinate whether Pyk2 phosphorylation is increased in cells transfected whit the FGFR3-SADDAN mutant.
For this purpose, immunoprecipitation experiments with specific anti-phosphotyrosine antibody were performed.
First, immunoblot experiments with anti-HA antibody were performed to determine the phosphorylation level of the FGFR3 variants. As expected, only the SADDAN and SADDAN-754 receptor are recognized. Most intriguingly, the SADDAN-754 double mutant exhibits both the 120kDa immature and the 130kDa mature forms that present both a lower phosphorylation level respect the SADDAN receptor (Fig.6B, upper panel).
By re-probing the filter with anti Pyk2 antibody we determine the net increase of Pyk2 phosphorylation in cells transfected with the SADDAN mutant.
However, the increase Pyk2 phosphorylation, although to lesser extent, is also observed also in the SADDAN-754 double FGFR3 mutant.
We hypothesized that the Pyk2 increased phosphorylation could occur by the interaction with the

mature 130kDa FGFR3 on cell surface.

In addition, these results indicate that PLCγ has a key role only for Pyk2 recruitment by the immature ER-resident FGFR3 form.

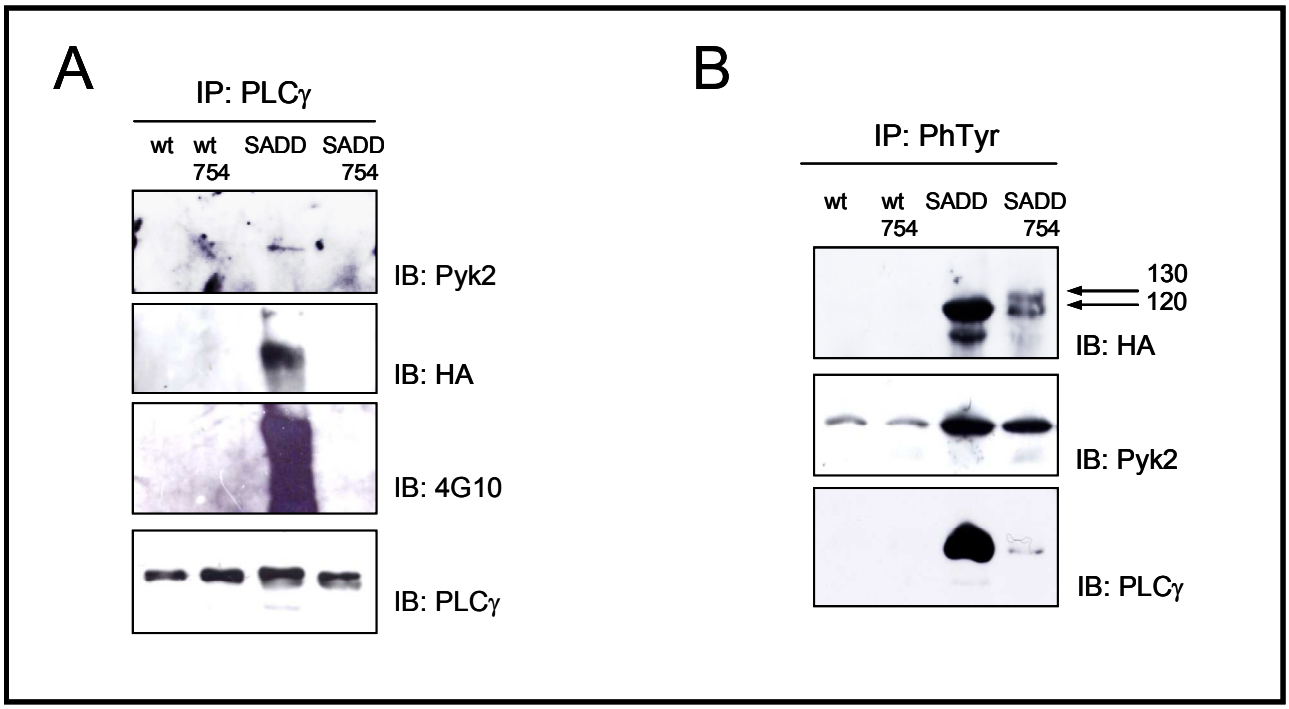

Fig.6 SADDAN receptor recruit PLCγ and Pyk2 from ER. (A) HEK293 cells were transiently trasfected with the HA-tagged wt, wt-754, SADDAN and SADDAN-754, were lysed and immunoprecipiteted with PLCγ antibodies. The immonocomplexes were analysed with anti-Pyk2, anti-HA, anti-4G10, anti-PLCγ antibodies as indicated. Pyk2 can directly bind PLCγ only the SADDAN receptor. (B) SADDAN mutant increase Pyk2 phosphorylation. The same samples were trasfected, lysed and immunoprecipiteted with anti-phosphotyrosine antibody and immunocomplexes were analysed with anti-HA, anti-Pyk2 and anti-PLCγ antibodies as indicated.

2.2 Paxillin interacts with the ER core-glycosylated SADDAN-FGFR3 form.

Paxillin through the interaction of its multiple protein-binding modules, many of which are regulated by phosphorylation (44), function as a recruitment platform of a number of regulatory and structural proteins for the control of cytoskeletal organization and cell adhesion.

Taking into account these observations, we have investigated on the interaction between FGFR3 and cytoskeleton proteins.

At first we have preformed immunoprecipitation experiments to determinate the interaction between paxillin and the FGFR3 variants. Fig. 7A shows a strong interaction between paxillin and FGFR3. The most striking observation is that paxillin pulls down only the120kDa FGFR3 forms suggesting that the paxillin interacts only with the immature FGFR3 in transit through the ER/cis-Golgi. Surprisingly, paxillin interacts as well with the FGFR3-wt and wt-754 variant but it confirms that the interaction occurs only with the immature ER-resident 120kDa glycomers. These data

indicate that paxillin interacts with FGFR3 independently by the phosphorylation of the receptor and by the presence of PLCγ since both the wt and the wt-754 do not exhibit kinase activity and do not associate with PLCγ.

We also tested the interaction between vinculin, another important protein that mediates cytoskeletal organization, and FGFR3.

In Fig.7A is shown that vinculin pulls down both SADDAN and the SADDAN-754 double mutant however, vinculin pulls down both the 120 and 130kDa mature form of the FGFR3-SADDAN-754 variant indicting that the interaction with the FGFR3 occur at both cell surface and the ER.

Previous work showed that the substitution of lysine at position 502 abolished the receptor kinase activity (kinase dead, KD).

To determine whether the FGFR3 kinase activity plays a role on paxillin recruitment, experiments were performed on a SADDAN-kinase dead (KD) double mutant in which the kinase activity is abrogated by the N502A amino-acid substitution (17).

Fig. 7B confirms that paxillin pulls down only the 120kDa immature receptor forms since it has been previously shown that the complete biosynthesis occurs for both the SADDAN-KD (16) and of the SADDAN-754 e variants (see Fig.6B).

The same filter was re-probed with anti-Pyk2 antibody, we find that paxillin pulls down Pyk2 in association with both SADDAN and SADDAN-KD

The SADDAN-754 does not allow Pyk2 recognition by paxillin. These data indicate that the position of the receptor in the ER is a prerequisite for the formation of the signalling platform mediated by the FGFR3 variants.

The paxillin/Pyk2 interaction in cells transfected with wt and wt-754 FGFR3 variants results of a basal phosphorylation of paxillin.

Interestingly, PLCγ is not recognized in either case indicating that it does not directly participate in the paxillin-Pyk2 recruitment.

Our next aim was to determinate whether the role by PLCγ on the paxillin recruitment by FGFR3, we performed experiments in which cells trasfected with FGFR3 variants, were treated with the PLCγ enzymatic activity inhibitor U73122 (44).

U73122 is an aminosteroid that is reported to act as a specific inhibitor of phospholipase C and it has become an important tool in establishing the link between phospholipase C activation and cellular Ca2+ signalling; this drug can directly activate ion channels. It can itself promote the release of Ca2+ from intracellular stores in permeabilized cells and in intact cells it triggers a release of Ca2+ that is initiated specifically at the secretory pole of these morphologically and functionally polarized cells.

Indeed, Figure 7C shown that PLCγ activity is not required for paxillin recruitment by FGFR3.

More importantly, the treatment with endoglycosidase H (EndoH), which is the enzyme that removes the carbohydrate moieties only from proteins with high-mannose configurations residenting in ER, confirms that paxillin associates exclusively with the immature ER-resident FGFR3 glycomers. Following Endo H treatment the 120kDa species is reduced to a 98kDa corresponding to the native unmodified protein.

A further information obtained by the treatment with EndoH is that the 120kDa FGFR3-SADDAN results to be EndoH-resistant (middle panel) indicating that by abolishing the PLCγ activity, The SADDAN receptor modifies its glycosilation pathway and receptor may not be in the high-mannose configuration suggesting that its position may be further downstream in its secretory pathway. This observation is in line with the fact that the biosynthesis of the SADDAN-754 mutant presents a full maturation pattern (see Fig.6B).

Our next effort was investigate whether paxillin exhibited an increase tyrosin- phosphorylation upon interaction with the FGFR3-SADDAN protein.

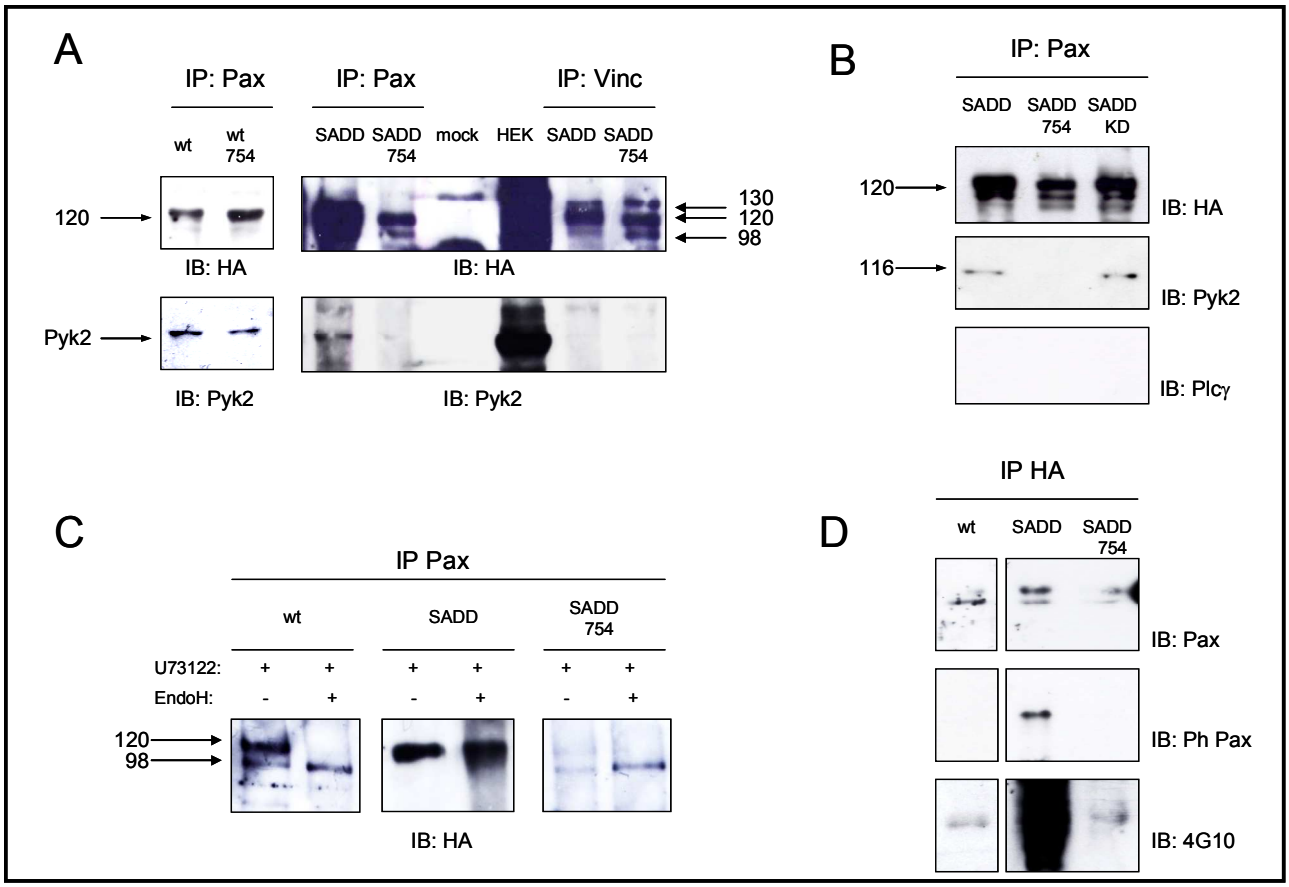

Fig.7 Paxillin binding the ER-resident FGFR3 forms, and this interation is independent from phosphorylation of FGFR3, binding and enzymatic activity of PLCγ. Paxillin not recruit Pyk2 in SADDAN-754 mutant, which bind Pyk2 with the mature 130kDa form. Vinculin recruit the 130kDa and 120kDa FGFR3 isoform. FGFR3-SADDAN ncrease level phosphorylation of paxillin. (A), (B) Upon transfection, the indicated lysates were immunoprecipitated with anti-paxillin or anti-vinculin. The immunocomplexes were analysed with anti-HA, anti-Pyk2 and anti-PLCγ antibodies. (C) The samples were treatated with U73122 for 24h., upon transfection, and immunoprecipitated with anti-paxillin antibody. The immunocomplexes were deglicosylated with EndoH and analysed by immunoblot with antibody detecting anti-HA. (D) The indicated lysated were immunoprecipitated with anti-HA antibodies followed by immunoblot with paxillin, anti-Ph-paxillin and anti-4G10 antibodies to shown the level of phosphorylation of paxillin.

receptor.

By re-probing the filter with the pan-phospho-tyrosine antibody 4G10, it is shown that paxillin exhibit a basal level of phosphorylation in line with previous reports (31) and such phosphorylation is dramatically increased in correspondence with the SADDAN receptor.

2.3 Disorganization of the actin cytoskeletal in cells expressing the SADDAN receptor.

Previous studies have shown that the effect of FGFR3 signalling on mature chondrocytes determines growth arrest accompanied by profound changes in cytoskeletal organization

Basal tyrosine phosphorylation of focal adhesion proteins is essential for their formation and maintenance. However, increased tyrosine phosphorylation of focal adhesion can disrupt these structures.

We observed that the phosphorylation of paxillin/Pyk2 is dramatically increased in correspondence with the SADDAN receptor.

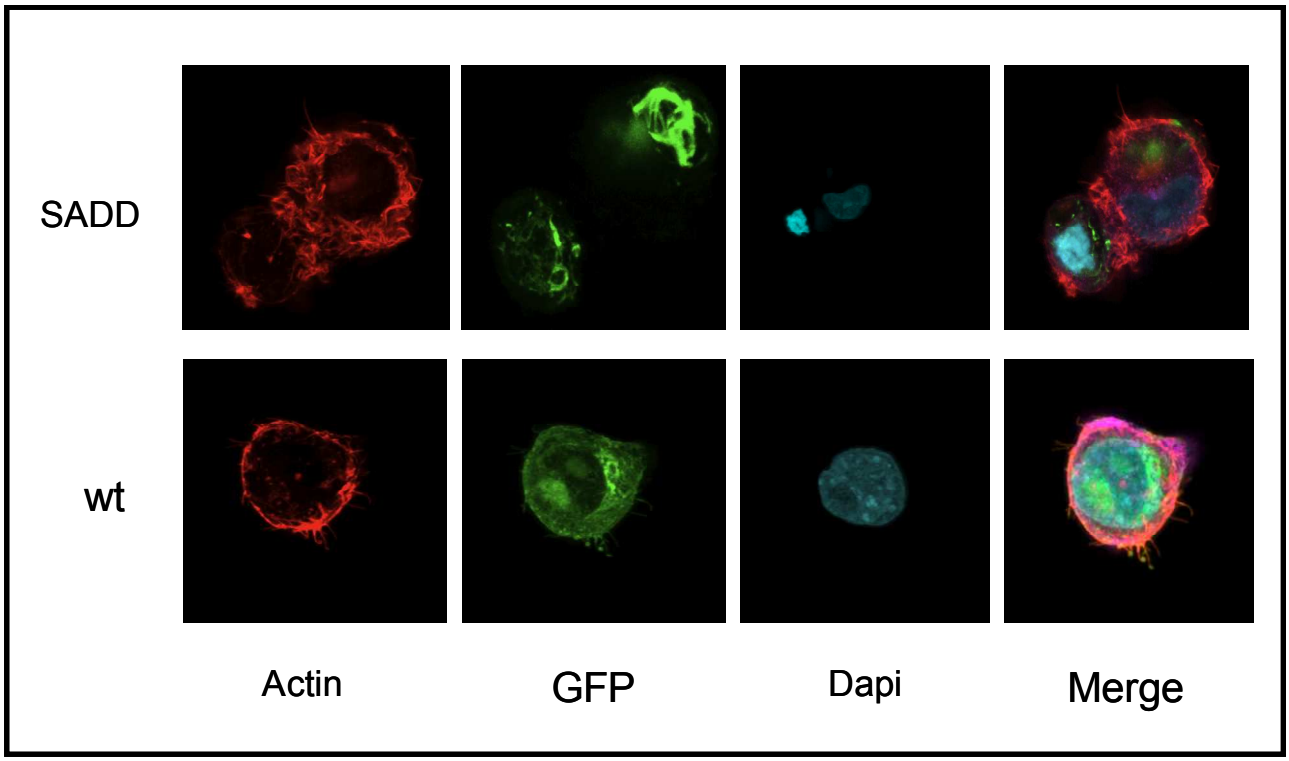

Fig.8 The actin is a target protein to study the organization of the cytoskeleton. HEK293 cells transfected with FGFR3-SADDAN or wt were plated on dishes and labeled with anti-actin (red), anti-GFP (green) antibodies. We are analyzed by confocal microscopy and the cells transfected with the mutant SADDAN show the actin cytoskeleton disorganized.

To determine whether FGFR3-SADDAN affects the cytoskeletal organization, confocal analysis with a specific anti-actin antibody was performed.

HEK293 cells were transfected with FGFR3-wt and SADDAN, the cells were labelled with anti-

GFP and anti-actin antibodies after plating onto dishes.

Fig.8 shown the cells analyze by confocal microscopy.

The cells transfected with the mutant SADDAN are more polarized and actin (in red in fig.8) appears to be disorganized compared to that of cells expressing FGFR3-wt.

These results suggest that the anomalous signalling of FGFR3-SADDAN from ER leads to a defect in the formation of focal adhesion and membrane protrusions.

Subsequently, we studied by confocal microscopy assays the distribution of vinculin and paxillin in cells transfected with mutant SADDAN or FGFR3-wt.

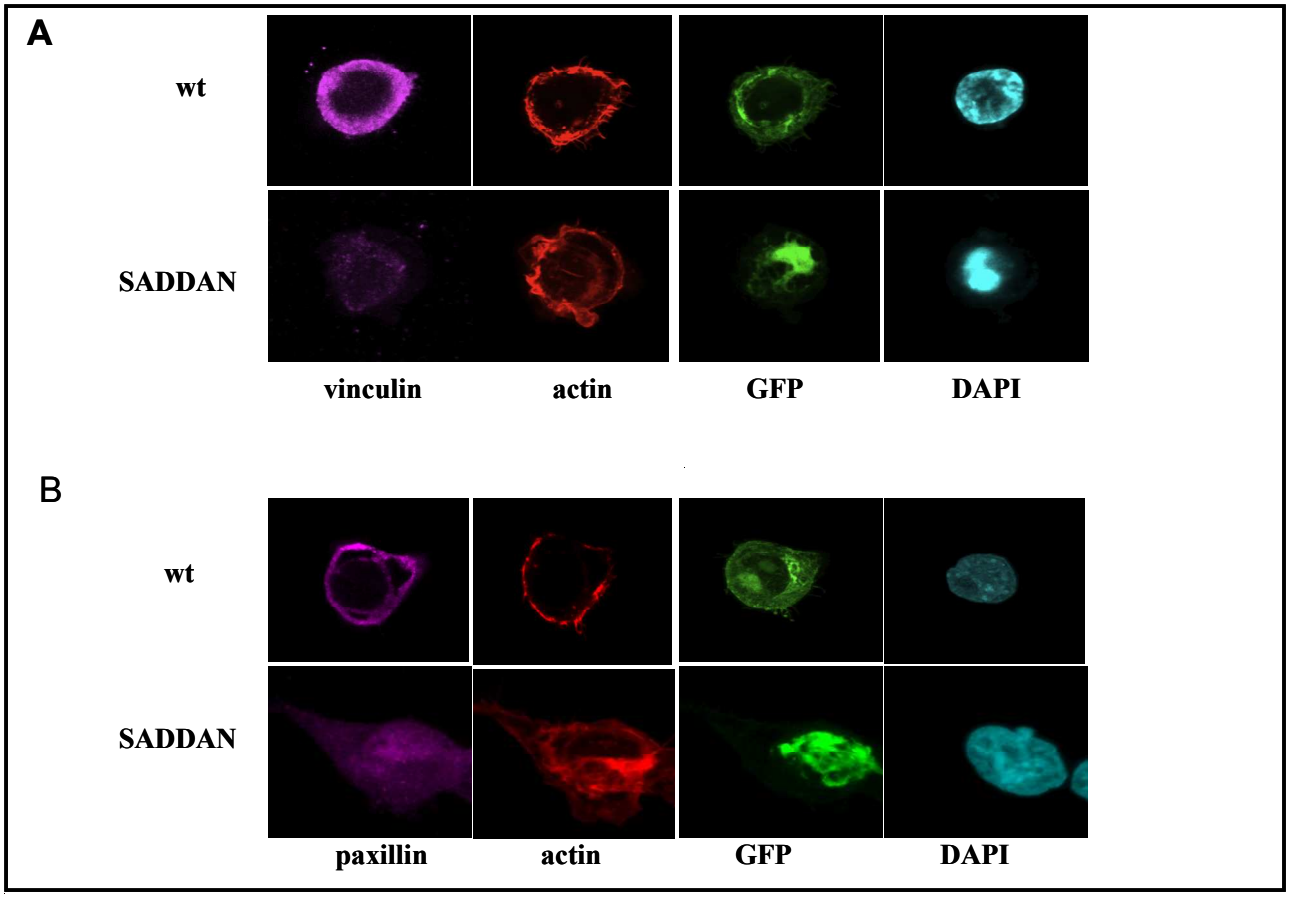

Fig. 9 Vinculin and paxillin are two proteins important for the organization of actin cytoskeleton. (A) HEK293 cells transfected with FGFR3-wt or SADDAN were plated on dishes and labeled with anti-actin (red), anti-GFP (green), anti-Vinculin (pink) antibodies. (B) HEK293 cells transfected with FGFR3-wt or SADDAN were plated on dishes and labeled with anti-actin (red), anti-GFP (green), anti-paxillin (pink) antibodies.
We are analyzed by confocal microscopy and in FGFR3-wt vinculin and actin are distribuited by membrane and is co-localized with actin. In SADDAN mutant it is alterated the distribution of thise proteins.

In FGFR3-wt vinculin and paxillin are distributed by membrane and is co-localized with actin (Fig.9A and B, upper panel), while in SADDAN it is evident that is altered also the distribution of vinculin and paxillin (Fig.9A and B bottom panel).

These data confirm our hypothesis that abnormal signaling of the mutant SADDAN in the early secretory pathway leads to alterations in cytoskeletal structures and changes the distribution of

vinculin and paxillin, which are proteins involved in the organization of actin.

2.4 Abrogation of the binding with PLCγ allows the full maturation of FGFR3-SADDAN.

We have previously shown that the high levels of kinase activity of the FGFR3-mutants cause incomplete biosynthesis that result with accumulation of the immature/mannose-rich, phosphorylated receptors in the endoplasmic reticulum.

We have report that FGFR3 mutants activate Erks from ER trough an FRS2α-indipendent pathway: instead, a multimeric complex by recruiting PLCγ, Pyk2 and Jak1 is formed.

Until now, the role by PLCγ on the retention in the ER of high-mannose-phosphorylated FGFR3 forms has not been considered.

In the course of these studies we have noticed that the double SADDAN 754 mutant present both the 120kDa and the mature fully glycosilated 130kDa FGFR3 forms, following immunoprecipitation with anti-phosphotyrosine or anti-vinculin (see Fig.6A and 7A).

To confirm the role of PLCγ on FGFR3-SADDAN maturation, we have determined the biosynthetic profile of the SADDAN-754 variant.

Fig.10 A clearly shown that the SADDAN 754 double mutant exhibit both 120kDa and 130kDa forms as well as the wt and the wt 754 mutant, while the 120kDa form is present in the SADDAN line.

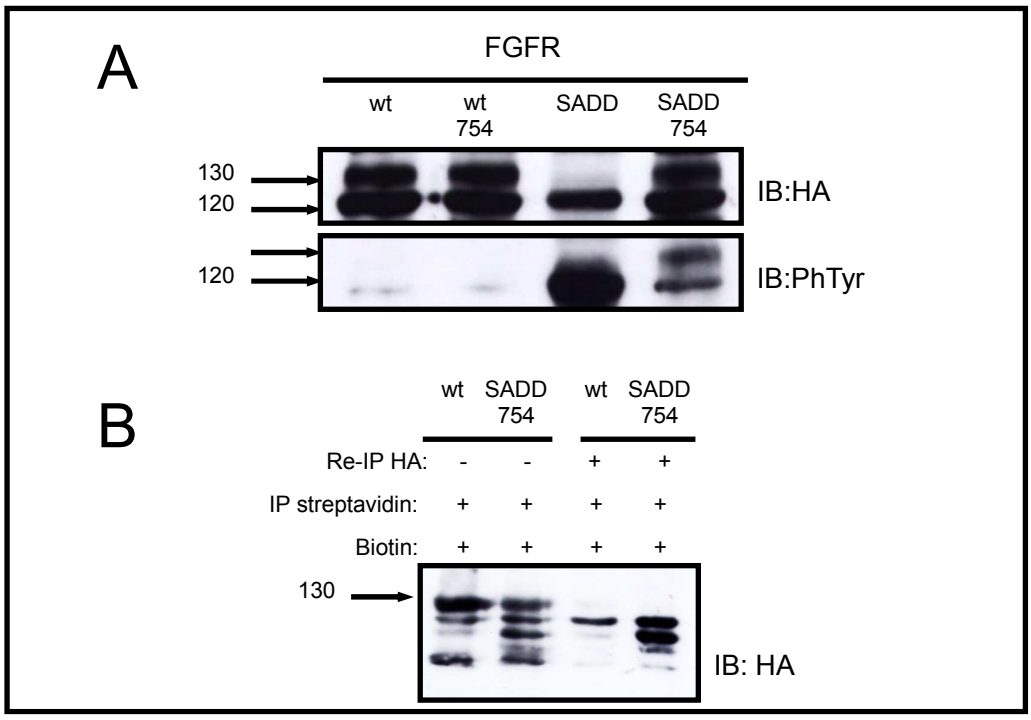

Fig.10 Abrogation of the binding with PLC allows the full maturation of FGFR3-SADDAN. (A) HEK293 cell were transiently transfected, lysed and analysed with anti-HA and anti-PhTyr antibodies. (B) Cell surface biotynilation of the SADDAN 754-FGFR3. Wt and SADDAN 754 cell were bitynilated for 10 min. The biotynilated proteins were recovered by streptavidin-agarose while the intracellular isoforms (un-biotynilated) were recovered by subsequent immunoprecipitation with anti-HA antibodies.

The full maturation pattern same is as well observed in the wt and the wt-754 mutant.
The same filter is re-probing with anti-phosphotyrosine antibody and as shown in Fig.6A the SADDAN 754 mutant preserves its tyrosine auto-phosphorylation, although to a lesser extent respect the one for SADDAN receptor.
To ascertain whether the mature SADDAN 754 130kDa form is exposed on cell surface, we preformed cell biotynilation assays, following by streptavidin immunoprecipitation experiments. As shown in Fig.10 B was it appears that the mature SADDAN-754 form is indeed exposed on the cell surface similarly to the mature wt receptor. A series of lower unspecific migrating bands are present as a probable unspecific biotinylation during preparation.
To further explore the role by PLCγ on SADDAN-FGFR3 maturation, experiments were performed with the U73122 inhibitor and U73433 inactive isomer, in the presence of endoglycosidase H (EndoH) an enzyme used to mark the protein position in the secretory pathway or PNGaseF. PNGase F is a an enzyme that removes all the carbohydrate moieties from proteins, following treatment the 130kDa and 120kDa species are reduced to 98kDa species corresponding to the native unmodified protein.
In Fig.11 is shown that in the presence of U73122 the SADDAN-FGFR3 become partially EndoH-resistent upon treatment.

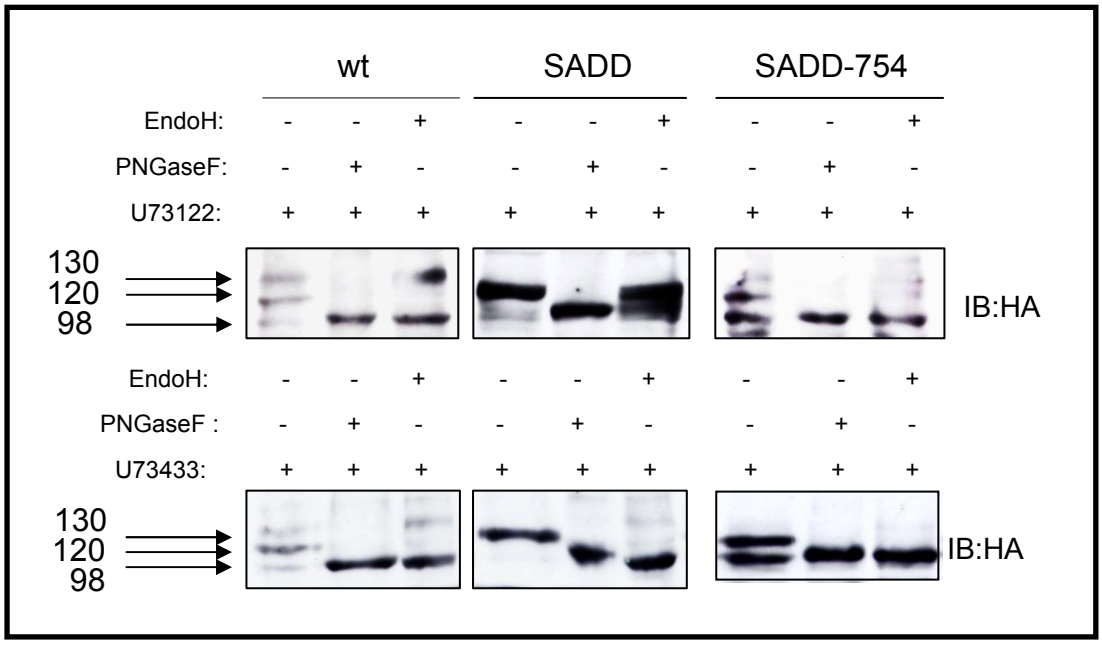

Fig. 11 Biosynthetic profile of FGFR3 species. Hek 293 cells were transiently transfected, trated with U73122 or U73433 for 24h and lysed. Samples were not trated, trated with EndoH or PNGaseF. Upon treatment with U73122, Saddan mutant become partially EndoH resistant.

This corroborates the notion that the immature 120kDa form is further processed downstream the

secretory pathway if the PLCγ activity is abrogated.

However, the mature 130kDa form does not appear in the FGFR3-SADDAN mutant upon treatment with U73122 indicating that the abrogation of PLCγ enzymatic activity by U73122 is not sufficient to accomplish the complete or the PLCγ activity is not totally abrogated in our system.

On the contrary, both SADDAN 754 and the wt-FGFR3 maintain the EndoH sensitive 120kDa form upon treatment with U73122 indicating their high-mannose composition in the ER/cis Golgi compartments is not perturbed by inhibition of PLCγ enzymatic activity.

Experiments repeated in the presence of the inactive isomer U73433 (Fig.11 lower panels) confirm the role played by PLCγ activity on FGFR3-SADDAN maturation.

Conclusion

In this study we have uncovered two events associated to PLCγ in relation to the highly activated FGFR3-SADDAN mutant. The first one deal with the signal transduction pathway triggered by the immature receptor from the ER, the second one is related to the biosynthesis/maturation of FGFR3-SADDAN.

The rational for this investigation was based on our studies in witch we have demonstrated that the highly activated FGFR3 with the K650M substitutions transducers the signal in its immature form from the ER. The high levels of kinase activity of the FGFR3-mutants cause uncompleted biosynthesis that results in the accumulation of the immature/mannose-rich, phosphorylated receptors in the endoplasmic reticulum (ER).

We report that FGFR3-SADDAN from the ER a multimeric complex by directly recruiting PLCγ and Pyk2 is formed. (17)

PLCγ is a key player in integrin-mediated cell spreading and motility achieved by the activation of Pyk2/paxillin/Rac1 signalling.

The mutant SADDAN recruits and increases the phosphorylation of Pyk2 trough binding and activation of PLCγ from ER.

The paxillin exhibit a basal level of phosphorylation in line with previous reports (31) and we have demonstrated that paxillin phosphorylation is increased following the interaction with the SADDAN receptor.

PLCγ does not directly participate in the paxillin/Pyk2/SADDAN recruitment and the inhibition of PLCγ enzymatic activity by U73122 affects the multimeric complex formation.

Increased tyrosine phosphorylation of focal adhesion can disrupt these structure, thus we have observed the cytoskeleton actin in HEK293 transfected whit wt and SADDAN receptor.

These data confirm our hypothesis that abnormal signaling of the mutant SADDAN in the early secretory pathway leads to alterations in cytoskeletal structures and changes the distribution of vinculin and paxillin, which are proteins involved in the organization of actin.

We have report that the highly levels of kinase activity of the FGFR3 mutants cause incomplete biosynthesis that results with of the immature/mannose rich, phosphorylated receptors in the ER.

Preventing the PLCγ/FGFR3 interaction by Y754F substitution, it was a surprise to discover that the SADDAN-754 double mutant present both the 120kDa and the mature fully glycosylated 130kDa FGFR3 forms, which preserved the tyrosine kinase activity also in the absence of the binding whit PLCγ.

The biotynilation assay confirmed that the mature SADDAN-754 130kDa form is exposed on the plasma membrane.

More interesting is the partially SADDAN-EndoH resistance upon U73122 treatment, indicating their high-mannose composition in the ER/cis Golgi compartments is perturbed by inhibition of

PLCγ enzymatic activity, even if this is not sufficient to accomplish the complete full maturation of the receptor.

We propose a model (Fig. 12) in which PLCγ is critical for both the FGFR3-perturbed cytoskeletal organization from the ER through Pyk2-paxillin pathway and the FGFR3 trafficking.

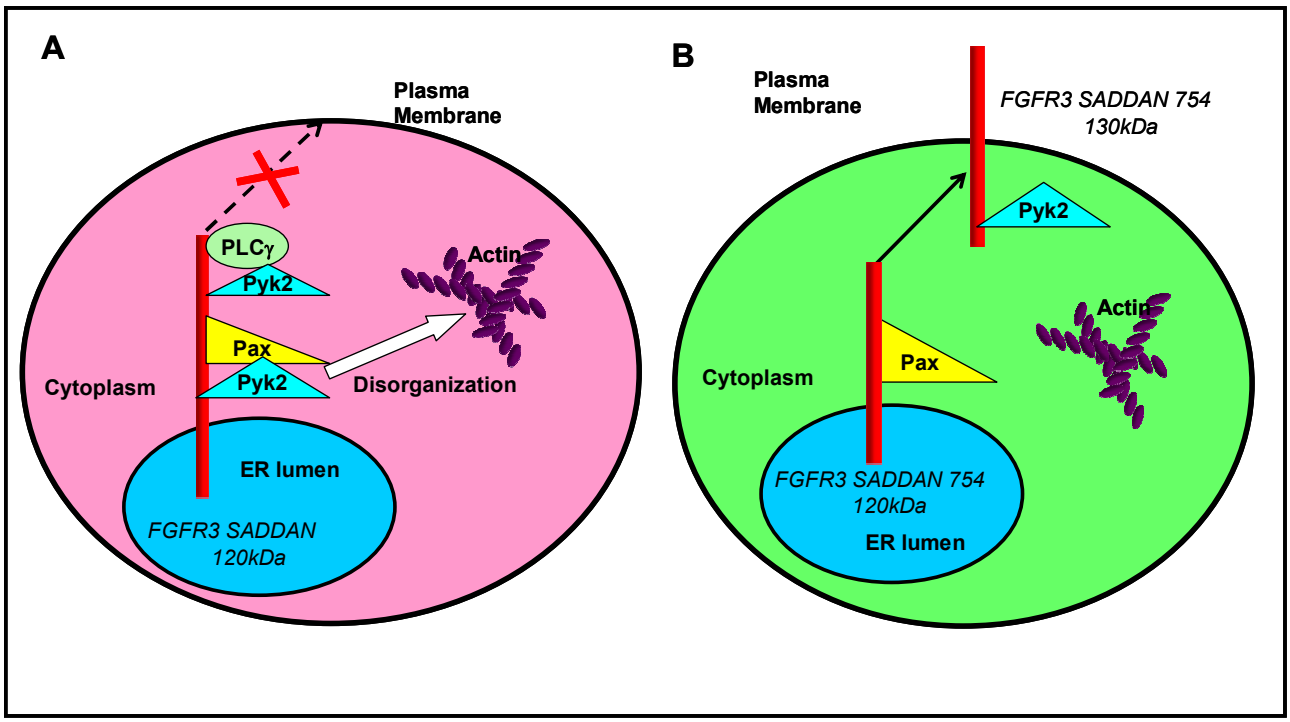

Fig.12 A schematic model describing signalin by mutant FGFR3. (A) The highly activated K650M FGFR3 mutants are trapped in ER in their immature phosphorylated form. FGFR3-SADDAN recruit PLC, Pyk2, Paxillin and disorganized actin cytoskeleton. (B) The double mutant SADDAN 754 present immature and mature phosphorylated forms in the ER and cell surface respectively. FGFR3-SADDAN 754 recruit Pyk2 from the cell surface.

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